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(21) International Application Number: PCT/EP97/02379 (22) International Filing Date: 9 May 1997 (09.05.97) (30) Priority Data: MI96A000956 10 May 1996 (10.05.96) IT (71) Applicant (for all designated States except US): INALCO S.P.A. [IT/IT]; Via Goldoni, 11, I-20129 Milan (IT). (72) Inventors; and (75) Inventors/Applicants (for US only): ZOPPETTI, Giorgio [IT/IT]; Via Mac Mahon, 43, I-20155 Milan (IT). ORESTE, Pasqua [IT/IT]; Via Mac Mahon, 43, I-20155 Milan (IT). CIPOLLETTI, Giovanni [IT/IT]; Via Tagliamento, 21, I-20139 Milan (IT). (74) Agent: GERVASI, Gemma; Notarbartolo & Gervasi, Corso di Porta Vittoria, 9, I-20122 Milan (IT).		(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
(54) Title: DERIVATIVES OF K5 POLYSACCHARIDE HAVING HIGH ANTICOAGULANT ACTIVITY		
(57) Abstract Derivatives of the K5 polysaccharide having high anticoagulant activity obtained by a process comprising the N-deacetylation of the K5 polysaccharide followed by N-sulfation, epimerization, O-sulfation and in case by N-resulfation.		

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DERIVATIVES OF K5 POLYSACCHARIDE HAVING HIGH ANTICOAGULANT ACTIVITY

PRIOR ART

It is known that the product mainly used in anticoagulant therapy is the heparin obtained by extraction from animal organs.

5 However the production of heparin from animal organs employs great amounts of solvents and chemical agents involving disposal and therefore potential environmental pollution problems. Moreover the final product may contain residues of biological substances normally or exceptionally present in the animal tissues as viruses or prions.

10 O-sulfation processes carried out on derivatives of the K5 polysaccharide (B. Casu et al., Carbohydrate Letters, 1, 107-114 (1994)) are also known.

The publications by B. Casu et al. refer to O-sulfations carried out on K5 which result in products showing an anticoagulant strength lower
15 then the commercial heparin.

This is also due to the fact that the entirety of uronic acids is represented by glucuronic acids. The glucuronic acid gives to the polysaccharidic chain a lower flexibility towards the target proteins such as for example the antithrombin III and then a lower
20 anticoagulant activity (B. Casu, M. Petitou, M. Provasoli and P. Sinay (1988). Conformational flexibility: a new concept for explaining binding and biological properties of iduronic acid containing glycosaminoglycans. Trends Biochem. Sci. 13, 221-225).

SUMMARY

25 Now a process for the preparation of new derivatives of the K5 polysaccharide which allow to overcome the drawbacks of the prior art

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has been found.

Said process includes the following steps:

- a) the K5 polysaccharide is N-deacetylated;
 - b) the product obtained in the step a) is N-sulfated;
 - 5 c) the product obtained in the step b) is epimerized to the achievement of at least 50% of iduronic acid with respect to the total of uronic acids,
and it is characterized in that the product obtained in the step c) is further treated according to the following steps:
 - 10 d) the product obtained in the step c) is dissolved in water and percolated through a column containing a cation exchange resin;
 - e) the solution obtained in the step d) is reacted with an organic base;
 - f) the solution obtained in the step e) is freeze-dried and the
15 obtained product is redissolved in an organic solvent and treated with a sulfating agent to obtain the O-sulfation;
 - g) the product obtained in the step f) is precipitated, redissolved in distilled water and dialyzed against distilled water;
 - h) If it is necessary, the product obtained in the step g) is treated
20 with a sulfating agent in order to obtain the N-resulfation of the in case N-desulfated groups.
- Optionally the product obtained after the step h) is depolymerized by controlled nitrous acid degradation according to known technology (i.e. US Patent No. 5,019,649).
- 25 The products according to the present invention have, beside new characteristics, a high anticoagulant activity, greater than the anticoagulant activity of the heparin obtained by extraction from

animal tissues.

DETAILED DESCRIPTION OF THE INVENTION

The characteristics and the advantages of the derivatives of the K5 polysaccharide and the relative preparation process according to the present invention will be mostly pointed out during the following detailed description. The starting material for the achievement of said derivatives is the K5 polysaccharide obtained from E. Coli as described by M. Manzoni, S. Bergomi and V. Cavazzoni (Journal of Bioactive and Compatible Polymers. Vol. VIII, July 1993, 251-257).

10 The K5 polysaccharide is first of all treated as in the following steps:

- a) the K5 polysaccharide is N-deacetylated;
- b) the product obtained in the step a) is N-sulfated in order to obtain N-sulfated K5 from 25 to 100%;
- 15 c) the product obtained in the step b) is epimerized with D-glucuronyl L-iduronyl C5 epimerase extracted from bovine liver in order to obtain a product having a L-iduronic acid content from 50 to 90% with respect to the total of the uronic acids.

The N-deacetylation of the step a) is carried out by treatment with a hydrazine and hydrazine sulfate mixture or in an alkaline environment with sodium hydroxide or potassium hydroxide. Subsequently the N-sulfation of the step b) is carried out by treatment with triethylamine-sulfur trioxide or with trimethylamine-sulfur trioxide.

The N-deacetylation and N-sulfation reactions are carried out according to the known techniques, for example according to the Patent

25 WO 92/17507.

The N-sulfated product is then submitted to the epimerization reaction

of the step c) in order to convert the glucuronic acid in iduronic acid. The epimerization is carried out present the D-glucuronyl-L-iduronyl-C5-epimerase enzyme (hereinafter simply denoted with C5-epimerase) extracted from bovine liver and purified by the method
5 described by A. Malmström in J. B. C. 255, 3878-3883 (1980).

The reaction medium is a buffer solution at pH 7.4 for example consisting of HEPES 0.04 M or TRIS 0.05 M, potassium chloride, EDTA and TRITON X-100 and added with one or more additives selected from the group consisting of glycol, glycerol, polyvinylpyrrolidone,
10 particularly polyvinylpyrrolidone having a molecular weight from 15.000 to 90.000, glycol and lecithin in such an amount to increase the viscosity of the buffer solution to values ranging from 1.1 to 3 centistokes. In particular the reaction medium is prepared starting from a suitable buffer solution having pH 7.4; such as for example
15 HEPES 0.04 M, KCl 0.4 M and EDTA 0.06 M and to 25 ml of this solution from 100 to 1000 µl of TRITON X-100, from 0.5 ml to 60 ml of additive and distilled water to 100 ml are added. The polysaccharide to submit to epimerization is added to said reaction medium in amounts from 5 to 1000 mg per 100 ml obtaining the solution A. The C-5 epimerase is
20 dissolved separately in the same reaction medium above mentioned in amounts from 21 to 2000 µg per 100 ml obtaining the solution B. The solution B is added to the solution A in such a proportion to obtain a C-5 epimerase content from 1.5 to 15.000 µg per 100 ml of mixture to submit to epimerization.

25 The mixture is homogenized by stirring and heated at a temperature ranging from 30 to 40 °C in a constant-temperature chamber for a time ranging from 90 minutes to 15 hours.

The reaction is stopped heating the mixture at 100 °C for 5 minutes.

The product is purified through a DEAE-Sephacel column using $(\text{NH}_4)\text{HCO}_3$ or NaCl 0.05 M as buffer and eluting the product with buffer $(\text{NH}_4)\text{HCO}_3$ or NaCl 2 M.

- 5 The gathered fractions are desalted by Sephadex G-15 column, the fraction containing the product is freeze-dried and the product is analyzed by $^1\text{H-NMR}$. From the $^1\text{H-NMR}$ spectrum the D-glucuronic acid and the L-iduronic acid content is computed.

The obtained product may be redissolved in the solution A and treated
10 again with the solution B obtaining, with further epimerization treatments, an increase of the L-iduronic acid content.

The product obtained from the step c) as described above is further treated as described in the following steps, which characterize the present invention:

- 15 d) the product obtained in the step c) is dissolved in water and percolated through a column containing a cation exchange resin such as for example Amberlite IR 120 H^+ (Rohm and Haas) which is subsequently washed with distilled water.

The pH of the obtained solution ranges from 0.5 to 1.5;

- 20 e) the solution obtained in the step d) is treated with an organic base preferably selected from the group consisting of trimethylamine, triethylamine and tributylamine, dissolved in an organic solvent such as for example alcohol. The organic base amount added is such to obtain a solution pH ranging from 6.5 to 7.0. The organic base excess
25 is removed by treatment with diethyl ether;

f) the solution obtained in the step e) is freeze-dried and the obtained product is redissolved in an organic solvent at room

temperature and treated with a sulfating agent at a temperature ranging from -5 to 60 °C for a time period ranging from 10 to 24 hours, in order to obtain the O-sulfation.

Said organic solvent is preferably the anhydrous dimethylformamide and
5 said sulfating agent is preferably selected from the group consisting of pyridine sulphur trioxide, trimethylamine sulphur trioxide, triethylamine sulphur trioxide, tripropylamine sulphur trioxide and tributyl sulphur trioxide;

g) the solution obtained in the step f) is diluted with an equal
10 volume of water, a solution of NaOH at 4% is added to reach a pH equal to 9 and the product is precipitated by addition of 4 volumes of alcohol saturated by sodium acetate and maintaining the temperature from 3 to 5 °C for 10-15 hours. The obtained precipitate is dissolved in distilled water and dialyzed against distilled water in a 1,000
15 cut-off dialysis membrane for 3 days with extra-dialysis change every day;

h) if it is necessary, the solution obtained in the step g) is added with sodium bicarbonate to pH 9, it is heated at a temperature ranging from 50 to 60 °C and a sulfating agent selected from the group pointed
20 out in the step f) is added in order to obtain the N-resulfation of the groups in case desulfated during the treatment. This reaction is carried out under stirring for a period of time ranging from 5 to 10 hours, at a temperature ranging from 50 to 60 °C.

At the end the solution is desalted by a 3500 D dialysis against
25 decreasing solutions of NaCl for 5 days and the product is freeze-dried.

Optionally the product obtained after the step h) is depolymerized by

controlled nitrous acid degradation according to known technology (i.e. US Patent No. 5019649).

K5 polysaccharides derivatives having new characteristics and an anticoagulant activity greater than the heparin one obtained by the
5 extraction from animal tissues are obtained by the described process.

The derivatives according to the present invention contain from 40 to 100% of chains affine for the Antithrombin III, computed according to the method described by M. Hook et al. (FEBS letters, 66, 1976, 90-93), while the heparin contains only 30% of chains affine for the
10 Antithrombin III and this explains its greater anticoagulant activity.

In the following Table 1 the values of the chemical analysis and the anticoagulant activity in vitro of the derivatives according to the invention (A) in comparison with the commercial heparin (B) are reported.

TABLE 1

	(A)	(B)
Sulfates/carboxyls ratio	2.2-2.5	1.9-2.4
N-sulfates content	70% - 100%	86% - 91%
6-O-sulfates content	70% - 90%	64% - 89%
2-O-sulfates content	50% - 60%	71% - 78%
3-O-sulfates content	5% - 10%	0.5% - 2.0%
Chains affine for the Antithrombin III	40% - 100%	28 - 35
Anti-Xa	500 - 600	145 - 197
APTT	250 - 320	145 - 187

The sulfates/carboxyls ratio has been determined by the conductimetric method according to B. Casu et al. (Carbohydr. Res. 39.168 (1975)) while the sulfates distribution has been determined by nuclear magnetic resonance according to B. Casu et al. (Arzneim. 5 Forsch./Drug Res. 33(I), 1, 135-142 - 1983).

The 3-O-sulfates content has been determined by the method described by B. Casu et al. (Biochem J. 197, 1981, 599-609).

The anticoagulant activity has been measured as APTT according to L. Andersson et al. (Thrombosis Res. 9, 575 - 1976), and as Anti Xa 10 according to D. P. Thomas et al. (Thrombosis and Haemostasis 45, 214-1981).

Thanks to their characteristics the derivatives according to the present invention may be used for the preparation of pharmaceutical compositions suitable to the anticoagulant treatment in the human therapy.

Said compositions contain efficacious amounts of said derivatives in combination with pharmacologically acceptable excipients or diluents.

The posology for the human therapy is from 30 to 200 mg per day.

The derivatives according to the present invention also exhibit with
5 respect to heparin the great advantage to be viruses and prions free
and the production process has the advantage not to give polluting
effluents.

EXAMPLE 1

10 mg of 100% N-sulfated and 70% epimerized (that is containing 70% of
10 iduronic acid with respect to the uronic acids total) K5 have been
dissolved in 2 ml of water and put into an Amberlite IR 120H⁺ column
at room temperature. The column has been washed with 10 ml of water.
The eluate plus the washing liquid had a pH equal to 1.5. The solution
has been added with tributylamine to pH 5.5 using a solution of
15 tributylamine at 10% in ethanol. The excess of tributylamine not bound
to the polysaccharide has been removed by treatment with diethyl
ether. The solution has been finally freeze-dried.

Then the product has been redissolved in 3.2 ml of anhydrous
dimethylformamide at room temperature and 3 ml of anhydrous
20 dimethylformamide containing 0.153 g of piridine-sulphur trioxide have
been added. The obtained solution has been kept at room temperature
for 6 hours and then diluted with an equal volume of water. The pH has
been finally set to 9 with NaOH at 4% and the product has been
precipitated with 4 volumes of ethanol saturated with sodium acetate
25 keeping the solution at 4 °C overnight. The obtained precipitate has
been dissolved in 10 ml of distilled water and dialyzed against
distilled water in a 1.000 cut-off dialysis membrane for 3 days with

- 10 -

extra-dialysis change every day.

The obtained sample has been submitted to N-resulfation. The pH has been set to 9 with the addition of solid sodium bicarbonate, the temperature has been raised to 55 °C and 6.5 ml of trimethylamine-sulphur trioxide have been added under stirring. The solution has been kept at 55 °C for 1 hour, further 6.5 ml of trimethylamine-sulphur trioxide have then been added and the reaction carried on for additional 5 hours. The sample has been desalted by 3500 D dialysis against solutions having decreasing NaCl concentration for 5 days (0.5 M the first day, 0.2 M the second day, 0.1 M the third day and water the fourth and the fifth day). The product has been finally freeze-dried.

The obtained product exhibits a sulfates/carboxyls ratio equal to 2.5, 100% N-sulfates content, 80% 6-O-sulfates content, 60% 2-O sulfates, 10% 3-O sulfates, a fraction affine to the AT III equal to 100% and the following in vitro anticoagulant activities:

Anti-Xa 600 U/mg

APTT 310 U/mg

EXAMPLE 2

10 mg of 90% N-sulfated and 60% epimerized K5 have been treated as in the Example 1 with the difference that, after the N-resulfation, the product has been treated at a temperature equal to 40 °C with 10 ml of anhydrous dimethylformamide containing 0.51 g of pyridine-sulfur trioxide added under stirring.

After 2 hours further 10 ml of anhydrous dimethylformamide containing 0.51 g of pyridine-sulfur trioxide have been added and the reaction has been continued for additional 10 hours.

The product has been desalted as in the Example 1.

The obtained product exhibits a sulfates/carboxyls ratio equal to 2.4, 90% N-sulfates content, 100% 6-O sulfates content, 40% 2-O sulfates, 7% 3-O sulfates, a fraction affine to the AT III equal to 85% and the

5 following in vitro anticoagulant activities:

Anti-Xa 550 U/mg

APTT 290 U/mg

EXAMPLE 3

10 10 mg of 80% N-sulfated and 55% epimerized K5 have been treated as in the Example 1 with the difference that, after the N-resulfation, the product has been treated at a temperature equal to 65 °C with 10 ml of anhydrous dimethylformamide containing 0.51 g of pyridine-sulfur trioxide added under stirring.

After 2 hours further 10 ml of anhydrous dimethylformamide containing
15 0.51 g of pyridine-sulfur trioxide have then been added and the reaction has been continued for additional 5 hours.

The product has been desalted as in the Example 1.

The obtained product exhibits a sulfates/carboxyls ratio equal to 2.5, 80% N-sulfates content, 100% 6-O sulfates content, 65% 2-O sulfates,
20 5% 3-O sulfates, a fraction affine to the AT III equal to 70% and the following in vitro anticoagulant activities:

Anti-Xa 450 U/mg

APTT 270 U/mg

In the Table 2 the data relative to the characteristics of the
25 products of the Examples have been summarized, from which one may notice particularly that the anticoagulant activity decreases with the decrease of the iduronic acid content.

EXAMPLE 4

10 mg of the product obtained in the Example 3 are dissolved in 10 ml of distilled water and added with 0.34 mg of sodium nitrite.

Immediately the pH is brought to 2.5 with hydrochloric acid 0.01 N.

5 After 40 minutes the solution is neutralised with sodium hydroxide and the compound is recovered by precipitation with 3 volumes of ethanol and dried in a vacuum oven.

The compound obtained shows a sulphate/carboxyl ration of 2.2, N-sulphate content of 70%, 6-O sulphate content of 65%, 2-O sulphate content of 60%, 3-O sulphate content of 4%, an ATIII high activity fraction of 40% and the following in vitro anticoagulant activities:

Anti-Xa 200 U/mg

APTT 70 U/mg

TABLE 2

	Example 1	Example 2	Example 3	Example 4
Sulfates/carboxyls ratio	2.5	2.3	2.4	2.2
Mean molecular weight	14,000	14,000	14,000	5,000
N-sulfates	100%	90%	80%	70%
6-O sulfates	80%	90%	70%	65%
2-O sulfates	60%	50%	60%	60%
3-O sulfates	10%	7%	5%	4%
Iduronic Acid	70%	60%	55%	55%
Fraction having high affinity for AT III	100%	85%	70%	40%
Anti-Xa	600 U/mg	550 U/mg	500 U/mg	200 U/mg
APTT	310 U/mg	290 U/mg	250 U/mg	70 U/mg

In the Figures 2, 3, and 4 the data relative to the affinity for the antithrombin III for the products of the Examples 1, 2 and 3 are reported, while in the Figure 1 are reported, by comparison, the same data for the heparin.

5 In said Figures:

- mAbs 530 nm means milliabsorbance determined at 530 nm;
- LA means fraction having low affinity for the antithrombin III;
- HA means fraction having high affinity for the antithrombin III;

These data confirm what has been reported as commentary of the Table

10 2.

CLAIMS

- 1 1. Process for the preparation of derivatives of the K5 polysaccharide
- 2 comprising the following steps:
- 3 a) the K5 polysaccharide is N-deacetylated;
- 4 b) the product obtained in the step a) is N-sulfated;
- 5 c) the product obtained in the step b) is epimerized at least to a
- 6 iduronic acid content equal to 50% with respect to the total of uronic
- 7 acids, characterized in that the product obtained in the step c) is
- 8 further treated according to the following steps:
- 9 d) the product obtained in the step c) is dissolved in water and
- 10 percolated through a column containing a cation exchange resin;
- 11 e) the solution obtained in the step d) is reacted with an organic
- 12 base;
- 13 f) the solution obtained in the step e) is freeze-dried and the
- 14 obtained product is redissolved in an organic solvent and treated with
- 15 a sulfating agent to obtain the O-sulfation;
- 16 g) the product obtained in the step f) is precipitated, redissolved
- 17 in distilled water and dialyzed against distilled water;
- 18 h) the product obtained in the step g) is if necessary treated with a
- 19 sulfating agent in order to obtain the N-resulfation of the in case
- 20 N-desulfated groups and, optionally, the obtained product is
- 21 depolymerized by controlled nitrous acid degradation.
- 1 2. Process as claimed in claim 1, characterized in that the solution
- 2 obtained in the step d) has a pH ranging from 0.5 to 1.5.
- 1 3. Process as claimed in claim 1, characterized in that said organic
- 2 base used in the step e) is selected from the group consisting of
- 3 trimethylamine, triethylamine, and tributylamine.

1 4. Process as claimed in claim 1, characterized in that the amount of
2 organic base used in the step e) is such to obtain a solution pH
3 ranging from 6.5 to 7.0.

1 5. Process as claimed in claim 1, characterized in that said organic
2 solvent used in the step f) consists of anhydrous dimethylformamide.

1 6. Process as claimed in claim 1, characterized in that said sulfating
2 agent used in the step f) is selected from the group consisting of
3 pyridine-sulfur trioxide, trimethylamine-sulfur trioxide,
4 triethylamine-sulfur trioxide, tripropylamine-sulfur trioxide and
5 tributylamine-sulfur trioxide.

1 7. Process as claimed in claim 1, characterized in that said treatment
2 with a sulfating agent of the step f) is carried out at a temperature
3 ranging from -5 to 60 °C for a time period ranging from 10 to 24
4 hours.

1 8. Process as claimed in claim 1, characterized in that said
2 precipitation of the step g) is carried out diluting the solution
3 obtained in the step f) with an equal volume of water, setting the pH
4 to 9, adding 4 volumes of alcohol saturated with sodium acetate and
5 keeping the temperature from 3 to 5 °C for a time period ranging from
6 10 to 15 hours.

1 9. Process as claimed in claim 1, characterized in that said sulfating
2 agent used in the step h) is selected from the group consisting of
3 pyridine-sulfur trioxide, trimethylamine-sulfur trioxide,
4 triethylamine-sulfur trioxide, tripropylamine-sulfur trioxide and
5 tributylamine-sulfur trioxide.

1 10. Process as claimed in claim 1, characterized in that said N-
2 resulfation reaction of the step h) is carried out at pH 9, at a

3 temperature ranging from 50 to 60 °C for a time period ranging from 5
4 to 10 hours.

1 11. Derivatives of the K5 polysaccharide N-deacetylated, N-sulfated,
2 epimerized at least to the 50% of iduronic acid with respect to the
3 total of uronic acids, having:

4 Sulfates/carboxyls ratio	2.2-2.5
5 N-sulfates content	70% - 100%
6 6-O-sulfates content	70% - 90%
7 2-O-sulfates content	50% - 60%
8 3-O-sulfates content	5% - 10%
9 Chains affine for	40% - 100%
10 the Antithrombin III	
11 Anti-Xa	500 - 600
12 APTT	250 - 320

1 12. Pharmaceutical compositions suitable to the anticoagulant
2 treatment in human therapy consisting of effective amounts of
3 derivatives of the K5 polysaccharide as claimed in claim 11, in
4 combination with pharmacologically acceptable excipients or diluents.

1 13. Therapeutic method for the anticoagulant treatment in human
2 therapy consisting in the administration of a derivative of the K5
3 polysaccharide as claimed in claim 11, in an amounts from 30 to 200 mg
4 per day.

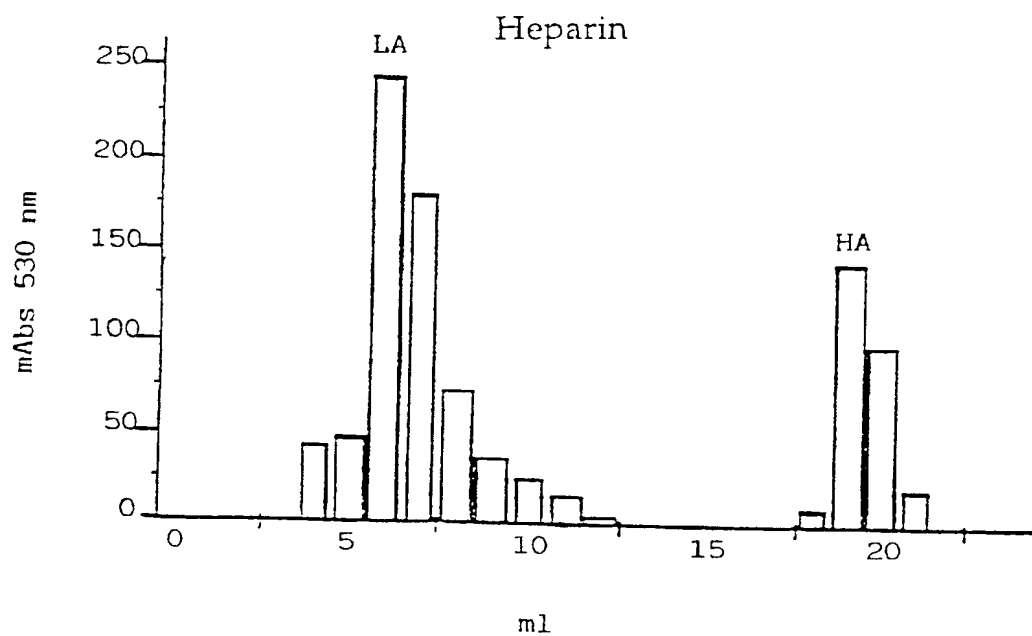


Figure 1

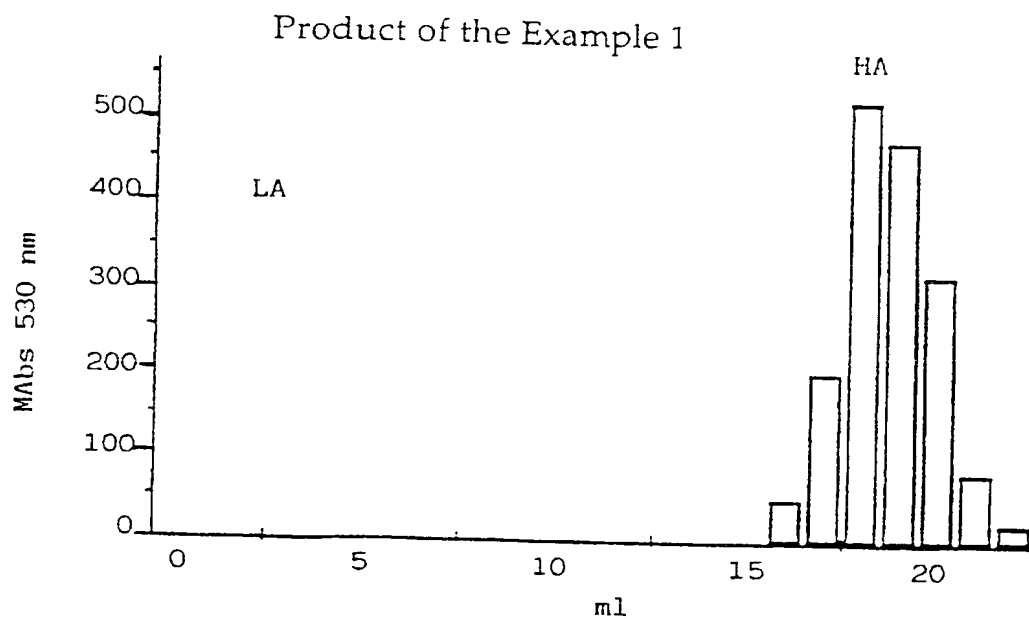


Figure 2

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Product of the Example 2

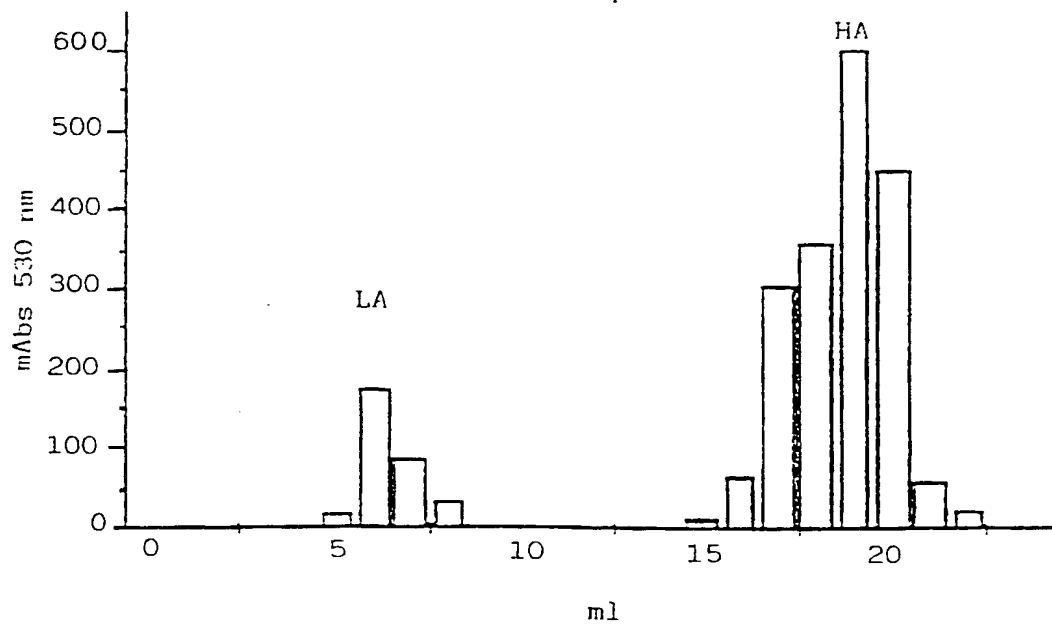


Figure 3

Product of the Example 3

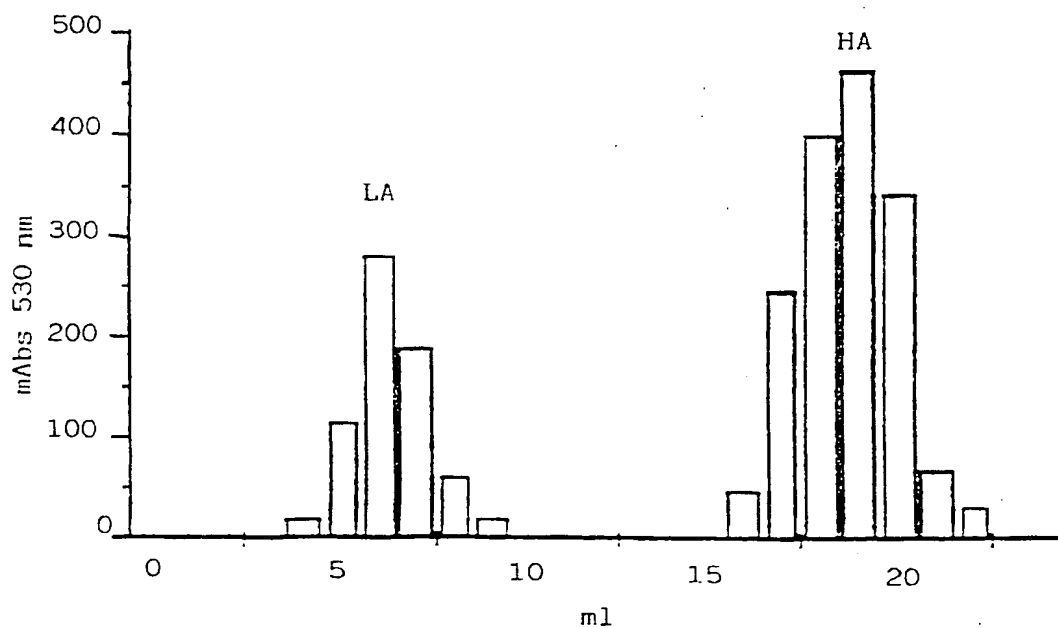


Figure 4

INTERNATIONAL SEARCH REPORT

International Application No.
EP 97/02379

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C08B37/00 A61K31/715

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C08B A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>WO 92 17507 A (ITALFARMACO SPA) 15 October 1992 cited in the application see page 12, line 13 - line 24 see claims</p> <p style="text-align: center;">-----</p>	1,11-13

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INTERNATIONAL SEARCH REPORT

International Application No
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Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9217507 A	15-10-92	GB 2254083 A	30-09-92
		AU 1430892 A	02-11-92
		EP 0577665 A	12-01-94
		GB 2286193 A	09-08-95
		HU 67208 A	28-02-95
		IT 1254564 B	25-09-95
		JP 7501684 T	23-02-95
		ZA 9202313 A	02-08-93

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